MITOTIC REPLICATION OF DEOXYRIBONUCLEIC ACID IN CHLAMYDOMONAS REINHARDI

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One of the central problems in molecular biology is to elucidate the replication mechanism of deoxyribonucleic acid (DNA) and its possible implication in genetic recombination. Of the several ingenious techniques that have been developed for the investigation of this problem,[1, 2, 3] the density gradient centrifugation technique developed by Meselson, Stahl, and Vinograd[4] provides the most promising method, and has already furnished the clearest picture of DNA replication in *Escherichia coli*.[5]

Here we have initiated a series of experiments in which the density gradient centrifugation technique has been used to investigate the mechanism of DNA replication in the chromosomes of an organism with clearly defined *mitosis* and *meiosis*. The unicellular green alga, *Chlamydomonas reinhardi*, was chosen as the experimental material because it possesses a number of advantageous characteristics. The alga can be grown easily in a minimal medium, the life cycle is extremely simple and yet it includes the characteristic features of higher organisms: vegetative growth, fertilization, and meiosis which can be readily controlled.[6] The chromosomes of *C. reinhardi* are well defined,[7, 8] and it has been established that the genetic segregation follows the classical meiotic pattern.[9, 10, 7] The present paper reports the mode of mitotic replication of *C. reinhardi* DNA. It is planned to report in a later paper the investigation of meiotic replication.

Isolation and Properties of DNA of C. reinhardi.—A wild-type, mating type plus strain of *C. reinhardi* was grown in a high salt minimal medium (Table 1) at 25°C

TABLE 1

Composition of Minimal Liquid Media

	Minimal* Medium	High Salt Minimal Medium
NH_4Cl, (gm)	0.05	0.50
$MgSO_4 \cdot 7H_2O$, (gm)	0.02	0.02
$CaCl_2 \cdot 2H_2O$, (gm)	0.01	0.01
K_2HPO_4, (gm)	0.72	1.44
KH_2PO_4, (gm)	0.36	0.72
Trace element solution,† (ml)	1	1
Distilled water, (liter)	1	1

* The minimal medium is the same as that of reference 10, except that NH_4Cl was substituted in place of NH_4NO_3.
† Hutner *et al.*[11]

in two-liter Erlenmeyer flasks. The cultures were aerated with five per cent carbon dioxide in air and stirred constantly by a magnetic stirrer. Illumination was from two sides and from above by Ken-Rad 30 watt daylight fluorescent lamps, which gave a light intensity of about 600 foot candles in the center.[12] Cell samples were withdrawn from a side siphon without opening the culture flask. When a cell concentration of 2×10^7 per ml was reached, the cells were centrifuged and washed once with a saline citrate solution (0.15 *M* NaCl plus 0.015 *M* sodium citrate, pH 7)

and suspended in 4 ml of the same solution for every 10^{10} cells. The cells were lysed by addition of sodium dodecyl sulfate (final concentration three per cent), and the DNA was purified by a chloroform-octanol method.[13] DNA preparations which contained three per cent protein by Folin-Ciocalteu method[14] have been obtained.

Both the purified DNA as well as DNA from the direct lysate were centrifuged in a 7.7 molal CsCl solution with calf thymus DNA as a density reference. It was found that the effective density of *C. reinhardi* DNA was, for both preparations, unusually high (1.728)[15] compared with that of DNA from phage, calf thymus,

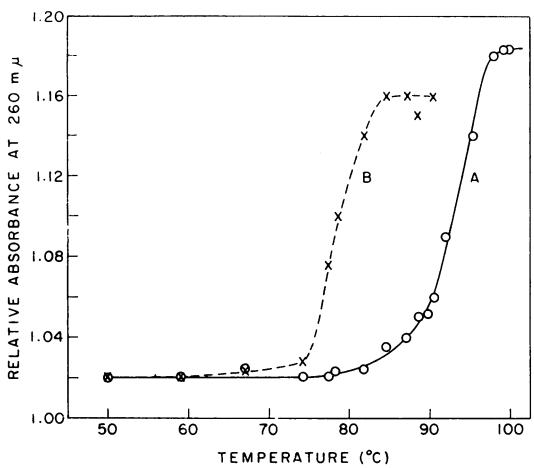


FIG. 1.—Hyperchromic shift of *C. reinhardi* DNA as a function of the temperature. Curve A: purified DNA of *C. reinhardi* was dissolved in the saline citrate solution (0.15 *M* NaCl plus 0.015 *M* sodium citrate, pH 7). Curve B: the same DNA in the saline citrate solution was dialyzed against a diluted saline citrate solution (0.015 *M* NaCl plus 0.0015 *M* sodium citrate, pH 7) at 3°C for 24 hours. The solution outside the dialysis tubing was changed three times and constantly stirred by a magnetic stirrer. The two DNA solutions in cuvettes were heated in the cuvette chamber of a Beckman DU spectrophotometer equipped with heating system,[16] and the optical densities at 260 mμ measured at various temperatures. The initial optical densities of the two solutions were 0.372 (A) and 0.223 (B).

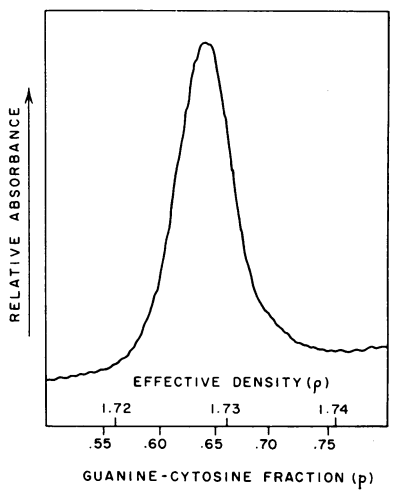


FIG. 2.—Distribution of purified *C. reinhardi* DNA in density gradient field. The ultraviolet absorption photograph of the equilibrated DNA band after 50 hours of centrifugation at 44,770 rpm (at 25°C) was traced by a Joyce-Loebl double beam microdensitometer. The density gradient at the peak position is 0.124 gm cm^{-3}. The guanine-cytosine scale was obtained from the equation $\rho = 0.103\ p + 1.662$[18], where ρ is the effective density and p is the molar fractional content of guanine and cytosine. The peak density is 1.728 and the apparent molecular weight calculated from the band profile is five million.

salmon sperm, and *E. coli* which range in density from 1.70 to 1.71. The fact that the purified DNA of *C. reinhardi* is not denatured is evident from a sharp hyperchromic shift obtained by heating it in the saline citrate solution (Fig. 1). The extremely high denaturation temperature of the DNA suggested a high guanine-cytosine content,[17] and, consequently, the possibility of a general correlation between the base composition and effective density of DNA was inferred. The latter possibility was explored by centrifuging DNA samples from various sources, and it was found that the effective density did increase linearly with the increase of the guanine-cytosine content.[18] According to this relationship *C. reinhardi* DNA

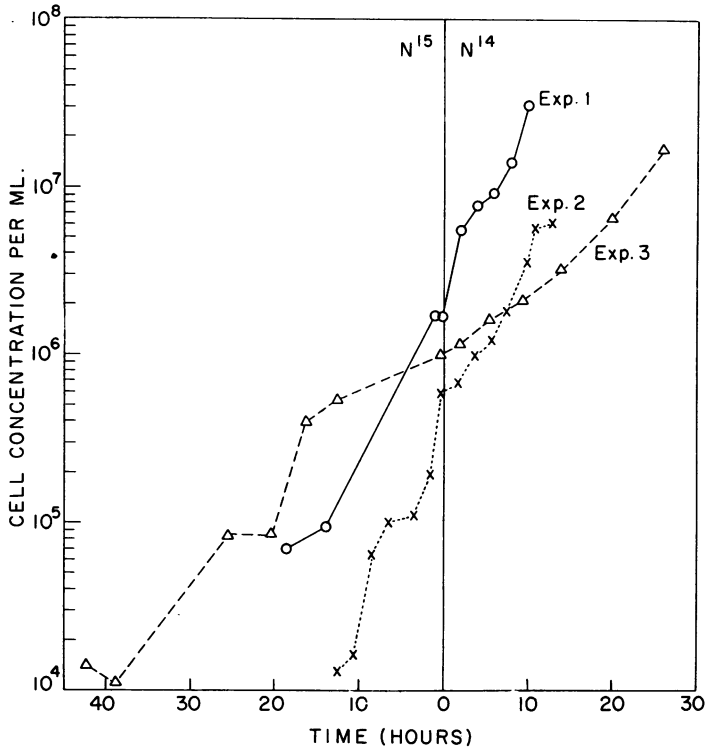


FIG. 3.—The growth curve of *C. reinhardi* in transfer experiments. The cell number was determined with the aid of a hemocytometer. The values on the ordinates have been corrected for the dilution by withdrawals of samples and addition of supplementing medium. At zero time the N^{15} medium was diluted with N^{14} supplementing medium. The medium used for Experiment 3 is different from that of the other two experiments in that the calcium content was reduced to half and 2 gm of sodium acetate per liter was added. The average generation time is about three hours in Experiments 1 and 2 and seven hours in Experiment 3. The error in the cell counting is usually within ten per cent. The stepwise growth curve comes from a partial synchrony of cell divisions and the characteristic growth habit of *C. reinhardi* in liquid culture (see Figs. 6 and 7).

should contain about 65 per cent guanine-cytosine.[19] A tracing of a band profile of the purified DNA is shown in Figure 2. Upon heating the DNA in the CsCl solution for 15 minutes at 100°C the effective density increases by 0.015 gm cm^{-3}. Preliminary results on other physical properties show that the sedimentation coefficient ($s_{20,w}$) of the purified *C. reinhardi* DNA in a solution of 0.15 *M* in NaCl and 0.015 *M* in sodium citrate at pH 7 is 23.4S and according to an empirical equation given in reference 20 corresponds to a weight average molecular weight of nine million. The apparent number average molecular weight calculated from the DNA band profile of density gradient centrifugation is five million. The discrepancy comes most likely from a reflection of the density heterogeneity which exists in the DNA sample and the difference between weight- and number-average molecular weights.[21]

Transfer Experiment.—Three transfer experiments from a nitrogen-15 (N^{15}) medium to a nitrogen-14 (N^{14}) medium have been made under slightly different con-

ditions. Since the results of each experiment were essentially the same, only one will be described here (Experiment 1, Fig. 3).

A two-liter flask containing 500 ml of minimal medium whose nitrogen was replaced by its heavy isotope, N^{15}, was inoculated with a suspension of fresh cells

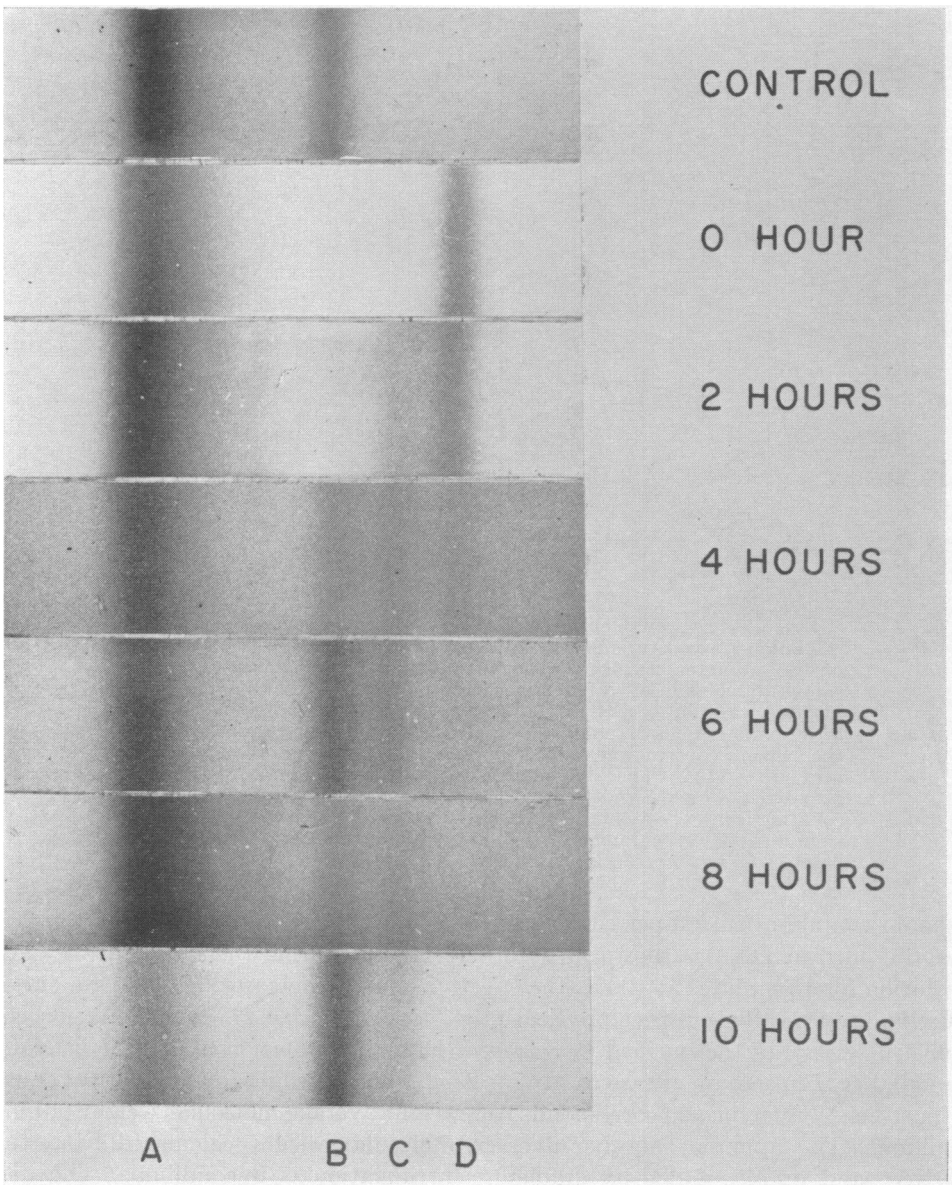


FIG. 4.—Ultraviolet absorption photographs of DNA bands of *C. reinhardi* lysates in density gradient field at various times after the dilution of N^{15}-culture by an excess amount of N^{14} substrates. The rotor speed was 44,770 rpm and the duration 20 hours at 25°C. Band A is calf thymus DNA added as a reference which enables an exact comparison with the other bands. In the first picture native DNA is shown as the control. The generation time is approximately three hours. Densities of the three *C. reinhardi* bands, non-labeled (B), half-labeled (C), and fully-labeled (D), are respectively 1.728, 1.735, and 1.742 at their peaks.

which had been grown on a minimal agar medium (two per cent agar). The initial concentration in the flask was 7×10^4 cells per ml. Samples of about 5 ml were withdrawn occasionally for making cell counts to determine the rate of growth. When the cell concentration reached 1.7×10^6 per ml, the first large aliquot (100 ml) was taken as the zero time sample. In order to replace N^{15} with N^{14}, a supplementing medium which contained ten times as much NH_4Cl as the minimal medium plus each of the four bases, adenine, thymine, guanine, and cytosine at concentration of 10 mg per liter was added to the culture bringing the total volume of the medium to 1.4 liters. Here the atomic dilution of N^{15} by N^{14} was 29 times. A sample of about 100 ml was taken every two hours and the same amount of the supplementing medium was added back to the flask. The growth curve throughout the transfer experiment is given in Figure 3. The cells of each sample were concentrated to about 5×10^8 per ml in a saline citrate solution (0.15 *M* NaCl and 0.015 *M* sodium citrate) and lysed with three per cent sodium dodecyl sulfate. An aliquot of the lysate (0.1 ml to 0.2 ml) was mixed in 0.8 ml of a nine molal CsCl solution. An appropriate amount of water was added to obtain desired density. Determination of the sample density was made by measuring the refractive index of the mixture of lysate and CsCl solution.[22] Following the addition of sodium dodecyl sulfate the protein and practically all pigments float to the surface of the mixture after it stands for a short time. The clear phase of the mixture is taken up by a syringe and put into the cell of the analytical ultracentrifuge. The density gradient was calculated for each experimental condition according to the procedure described by Meselson.[23] Ultraviolet absorption pictures of almost completely equilibrated DNA bands (after 20 hr at 44,770 rpm) of samples taken at various times after the transfer are shown in Figure 4, and the relative amount of DNA in each band at different times as estimated from tracings of the ultraviolet absorption photographs is shown in Figure 5. Figures 4 and 5 are the same experiments and are typical of the results obtained. There are three distinct kinds of molecules: heavy, intermediate, and light with the corresponding densities of 1.742, 1.735, and 1.728, respectively. The fair separation of these three bands indicates the absence of an appreciable pool of N^{15} DNA-precursors. The zero time picture shows that 95 per cent of DNA is heavy, indicating that the replacement of N^{14} with N^{15} is satisfactory. The succeeding pictures show that the transition of the bands is from heavy to intermediate then to light. It is clear that after two hours' incubation in N^{14}-medium (Fig. 4) some heavy DNA trans-

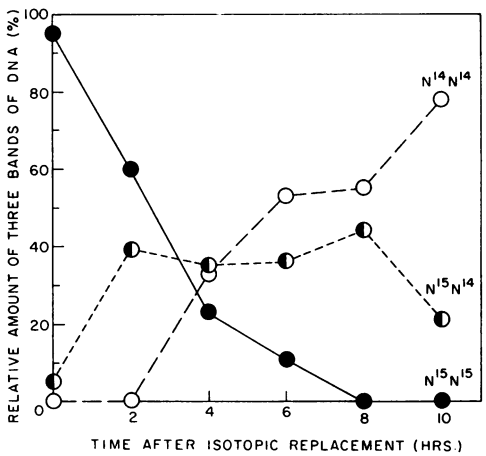


FIG. 5.—Transition of relative amount of fully-labeled ($N^{15}N^{15}$), half-labeled ($N^{15}N^{14}$), and non-labeled ($N^{14}N^{14}$) DNA after the isotope replacement. The relative amounts of the three kinds of DNA were measured from microdensitometer tracings of the ultraviolet absorption photographs whose positives are shown in Figure 4.

forms to intermediate DNA. The light DNA has not yet appeared at this time thus excluding the possibility of the total conservation of the old molecule in DNA duplication. The situation is exactly analogous to that of *E. coli*.[5] The transition of the bands, however, has a special feature, that is, there is a period at four and six hours after the transfer when all three bands are apparent. In *E. coli*, however, only the intermediate band is present at one generation after the isotope replacement. The possible cause of the difference in the transition of the bands in *C. reinhardi* and *E. coli* may lie in different characteristics of their growth, particularly with regard to nuclear division. Accordingly, the growth habit of *C. reinhardi* was analyzed in synchronized liquid cultures. The synchronized cultures were obtained by inoculating liquid minimal medium with fresh cells which had been grown under the light for two to three days on a minimal agar medium supplemented with 4 gm of yeast extract and 2 gm of sodium acetate per liter. The liquid culture was then incubated under the same conditions of light, temperature, and aeration as before. Samples of about 10 ml were withdrawn at various times and the cells immediately chilled to stop further growth. Cell counts and morphological observations were made after the cells had been immobilized with a minute amount of formaldehyde. The chilled cells were also taken to prepare slides for nuclear staining with azure-A.[24]

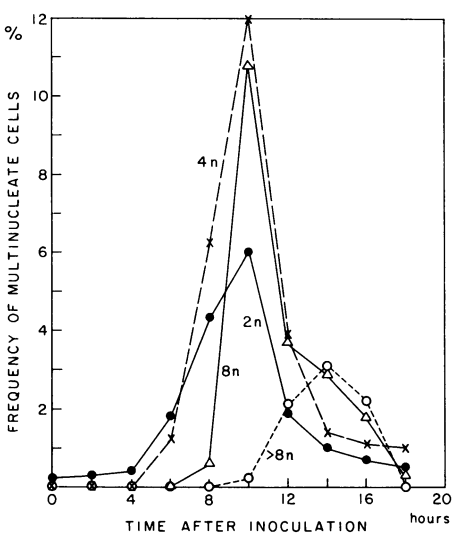


FIG. 6.—A typical transition of frequencies of multinucleate cells in the first cycle of a synchronized liquid culture. Fresh cells from two-day-old yeast agar plates were inoculated in a minimal liquid medium with the initial concentration of 6.4×10^4 cells per ml. For the conditions of culture, see the text. A drop of concentrated cell suspension was smeared on a slide which had been spread with a small amount of egg albumin. The fixation and staining of the preparation were made according to DeLamater.[24] In the figure, 2n, 4n, and 8n indicate the number of nuclei per cell, and >8n means the cell contains more than eight nuclei (mostly 16 and 32). Some of eight nucleate cells in the figure are in the C-colony stage (Fig. 5).

From the cytological observations of stained cells it is clear that before cell division occurs the cell undergoes a number of nuclear divisions. The frequency of cells with two, four, and eight nuclei is given in Figure 6. Figure 6 shows that before certain cells initiate a nuclear division, other cells have already undergone more than three nuclear divisions. It is quite conceivable that a similar situation is also true for DNA duplication. Consequently there is a period when both unduplicated DNA (heavy) and duplicated DNA (intermediate and light) are present thus forming the three bands simultaneously.

Observation of immobilized cells confirms this cytological finding. In order to analyze the cell variations in size and shape, intact cells were arbitrarily classified into three types; A-cells which are about 5 μ–6 μ in diameter, B-cells which are one of a group of cells 7 μ–14 μ in diameter, and C-colonies which consist of a group of unseparated cells from a single mother cell. The A-cells are apparently derived

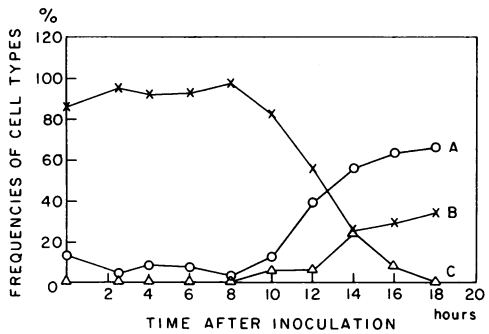
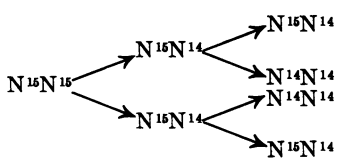


FIG. 7.—A typical frequency transition of cell types of *C. reinhardi* during the first division cycle in a synchronized liquid culture. A: Small cells, 5–6 μ in diameter. B: Large cells: 7–14 μ in diameter. C: Colonies of daughter cells originating from single B-cells. The culture is the same one described in Figure 6.

from separation of the cells of C-colony. The frequency of the occurrence of the different cell types with time is shown in Figure 7. It is clear that following separation of C-colonies the cells gradually increase in volume and then suddenly undergo cell divisions yielding eight or more daughter cells. The over-all picture of the growth pattern of *C. reinhardi* in liquid culture is shown schematically in Figure 8.

This characteristic growth pattern seems to be rather common in Chlamydomonas, since a similar pattern has been observed on agar cultures of *C. moewusii* and *C. eugametos*.[7]

Discussion.—The appearance of the three bands in the N^{15}–N^{14} transfer experiments is best interpreted by the bipartite structure of DNA, and the sequence of the transition among the three states of DNA, fully-labeled ($N^{15}N^{15}$), half-labeled ($N^{15}N^{14}$), and nonlabeled ($N^{14}N^{14}$) is most likely as follows,



This is the model which was proposed by Watson and Crick[25] and described as semi-conservative by Delbrück and Stent.[26]

The transfer experiment on mitosis of *C. reinhardi*, therefore, gives a pattern of DNA replication analogous to that of *E. coli*.[5] This pattern is one in which each continuous half of the DNA subunit is conserved in two daughter molecules of the succeeding generation. The results presented here generalize the finding of

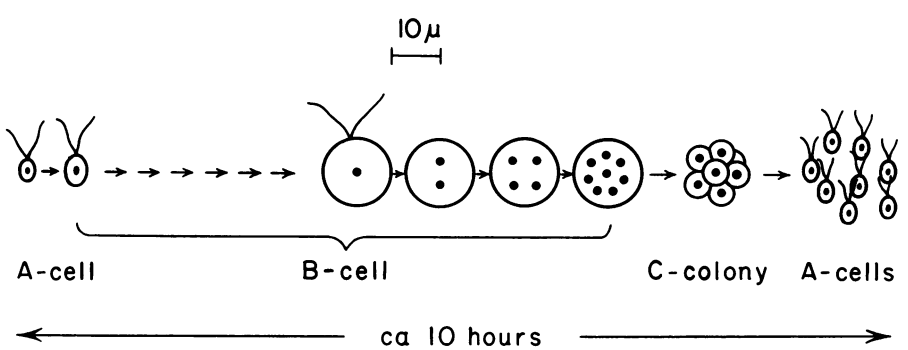


FIG. 8.—Schematic description of one vegetative growth cycle of *C. reinhardi* in minimal liquid culture. The nucleus is shown as a black dot.

Meselson and Stahl for *E. coli* to the chromosomes of higher organisms. In addition the results are formally consistent with the radioautographic analysis of chromosomes by Taylor, Woods, and Hughes.[3]

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[15] The density (1.728) was determined by reference to the peak density (effective density obtained from the position of the peak of DNA distribution in density gradient field) of calf thymus DNA which is 1.706. Reproducible values of density of any DNA to the third decimal place can be obtained by mixing the DNA with a reference DNA of known density and by calculating the density by means of the distance between the two bands of the reference DNA and the DNA in question. By this means the following standard peak densities have been obtained.

Diplococcus pneumoniae DNA	1.703
Calf thymus DNA	1.706
Escherichia coli DNA	1.713
Chlamydomonas reinhardi DNA	1.728

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*GENETIC STRUCTURE OF RECOMBINANT CHROMOSOMES FORMED AFTER MATING IN ESCHERICHIA COLI K12**

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Genetic studies on bacteria and bacteriophage have demonstrated the presence of a linear array of hereditary determinants.[1-3] Experiments on bacterial transformation and phage infection identify DNA (deoxyribonucleic acid) as the carrier of genetic information.[4-5] Replication of DNA has been studied with various organisms.[6-8] Experimental results suggest that DNA molecules are composed of two units, which separate from each other during duplication, to form two daughter molecules, each composed of a parental unit and of a newly synthesized one, and that the units themselves remain intact through succeeding duplications. They are consistent with the model of DNA duplication proposed by Watson and Crick.[9]

In chromosome duplication, the two units of the parental DNA, which are assumed to have equivalent genetic information, transfer the genetic characters of the parent to each of the two daughter chromosomes. However, when two chromosomes recombine, the DNA units of the two parents, which may not be identical, participate in the formation of recombinant chromosome(s). Therefore, the genetic structure of recombinant chromosomes may have characteristics different from those formed in a duplication process not involving recombination. In the present paper the genetic characteristics of recombinant chromosomes formed after mating in *E. coli* K12 are analyzed.

The process of bacterial recombination can be summarized as follows.[10-11] When a culture of *Hfr* cells (male, yielding a high frequency of recombination) and a culture of F^- cells (female) are mixed, some *Hfr* cells pair with F^- cells, forming an intercellular bridge. The *Hfr* chromosome is introduced into the F^- cell unidirectionally through the bridge. If in the course of transfer the chromosome breaks, the F^- cell receives only a fragment of the *Hfr* chromosome. After the transfer of genetic material, genetic recombination between the fragment of *Hfr* chromosome and the F^- chromosome takes place in the zygote and is followed by segregation.

Let us consider several models of the recombinant chromosome(s) formed after mating and examine the predictions made by each model as to the patterns of segregation to be observed in the descendants of a zygote. A bacterial chromosome